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(21) International Application Number: PCT/US97/17640 (22) International Filing Date: 17 September 1997 (17.09.97) (30) Priority Data: 714,864 17 September 1996 (17.09.96) US 799,171 14 February 1997 (14.02.97) US (60) Parent Application or Grant (63) Related by Continuation US 08/799,171 (CON) Filed on 14 February 1997 (14.02.97) (71) Applicant (for all designated States except US): AMUR PHARMACEUTICALS, INC. [US/US]; 1815 Old Country Road, Belmont, CA 94002 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): CHASALOW, Fred, I. [US/US]; 870 Crestview Drive, San Carlos, CA 94070 (US). (74) Agents: FRANKFORT, Howard, M. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PHOSPHOLIPID DRUG DERIVATIVES (57) Abstract <p>Disclosed herein are methods for increasing the bioavailability of pharmaceutical agents by conjugation to phospholipids. Also disclosed are phospholipid-derivatized steroids, peptides, antibiotics and other biologically active agents and pharmaceutical formulations comprising these compounds.</p>		

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PHOSPHOLIPID DRUG DERIVATIVES

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FIELD OF THE INVENTION

This invention pertains to methods and compositions for increasing the aqueous solubility and bio-availability of bioactive agents by conjugating them to phospholipids.

10

BACKGROUND OF THE INVENTION

Conventional means for delivering pharmaceutical and therapeutic agents to mammals often are severely limited by chemical and physical barriers to uptake, as well as by susceptibility of administered agents to rapid metabolic inactivation following uptake. Oral delivery of many biologically-active agents would be the route of choice if not for the extreme pH of the stomach, the action of proteolytic and other digestive enzymes in the intestine, and the impermeability of gastrointestinal membranes to the active ingredient.

Methods for orally administering vulnerable pharmacological agents have relied on co-administration of adjuvants (e.g. resorcinols and non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether) to artificially increase the permeability of the intestinal walls; co-administration of enzymatic inhibitors (e.g. pancreatic trypsin inhibitor, diisopropylfluorophosphate (DFP) and trasylol) to avoid enzymatic degradation; and encapsulation of the active agent in liposomes or other delivery vehicles.

Irrespective of the mode of administration of many therapeutic compounds, once they gain access to body tissues or fluids they are then subject to rapid inactivation in the liver, termed the first-pass effect. Orally administered compounds in particular are rapidly delivered to the liver via the portal circulation. Many compounds are acted upon by mixed-function oxidases, Phase I enzymes and other liver enzymes to produce inactive glucuronides, hippurates, glycyl and acetyl derivatives, which are rapidly excreted by the kidney.

There is thus a need in the art for methods and compositions to enable potential therapeutic agents to be rapidly absorbed in the intestine and avoid first-pass inactivation in the liver.

5 SUMMARY OF THE INVENTION

It has now been unexpectedly discovered that conjugation of many biologically active agents to phospholipid via a phosphodiester bond will significantly enhance the bioactivity and/or the bioavailability of such agents.

In one aspect, the present invention provides a method for increasing the
10 bioavailability of a pharmaceutical agent, comprising the steps of conjugating said agent to one or more phospholipid moieties, recovering said biologically active agent conjugated to said phosphocholine and administering said agent to a mammal wherein said agent in conjugated form is significantly more soluble in aqueous media than said agent in unconjugated form.

15 In yet another aspect, the present invention provides a composition of matter comprising an isolated phospholipid derivative of salicylic acid.

In yet another aspect, the present invention provides a pharmaceutical formulation for treating a mammal suffering from osteoporosis comprising an isolated phospholipid derivative of a compound selected from the group consisting of estrone or
20 estradiol and a pharmaceutically acceptable carrier or diluents.

In yet another aspect, the present invention provides a composition of matter comprising an isolated phospholipid derivative of an antibiotic selected from the group consisting of cephalosporin P1, fusidic acid and helvolic acid.

In yet another aspect, the present invention provides a composition of
25 matter comprising an isolated phospholipid derivative of dehydroepiandrosterone.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present description, claims and drawings.

DETAILED DESCRIPTION OF THE INVENTION

30 All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of inconsistencies, the present description, including definitions, will prevail.

Definitions

"Phospholipid-conjugated" or "phospholipid-derivatized" defined herein as covalently bonded to a phospholipid moiety via a phosphodiester linkage.

5 "Significantly enhanced bioactivity" or "significantly more soluble in aqueous media" in terms of the conjugated drugs of the present invention is defined herein as no less than 5 to 10-fold increased biological activity and/or aqueous solubility as compared to the unconjugated parent compound when administered by the same route.

The present invention is directed to increasing the bioavailability and/or aqueous solubility of pharmaceutically active agents, specifically by
10 conjugation of such agents to phospholipids, such as a phosphocholine moiety via a phosphodiester bond.

In accordance with the present invention, therapeutic substances will benefit by increasing their water solubility (and their bioavailability) by forming a phosphodiester between an (a) alcohol, and (b) a phospholipid. Non-limiting examples of the
15 phospholipid include phosphocholine, phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-phosphoethanolamine and N, N-dialkyl-phosphoethanolamine (all commercially available from Aldrich Chemical, Milwaukee, WI). Phosphocholine is particularly preferred as the phospholipid.

Phosphocholine is a ubiquitous component of biological membranes, usually
20 present in the form of phosphatidyl choline, i.e., attached via a phosphodiester bond to diacyl glycerol. The two most common phosphocholine-containing molecules are lecithin and sphingomyelin. Both of these compounds can be hydrolyzed by phospholipase C at the phosphocholine phosphodiester bond to release diacyl glycerol and ceramides, respectively. Importantly, both lecithin and sphingomyelin, which are present in food,
25 are absorbed in the gastrointestinal tract, incorporated into HDL-and LDL-cholesterol, and transported through the blood without significant first-pass metabolism in the liver.

In accordance with the present invention, conjugation of one or more phospholipid moieties to lipophilic compounds will render them more hydrophilic, without abrogating their ability to traverse biological membranes. Without wishing to be bound
30 by theory, it is contemplated that phospholipid conjugation will, in most cases, mask the biological activity of the conjugated compounds. The phospholipid conjugates will persist in conjugated form until they encounter enzymes such as phospholipase C, sphingomyelinase and non-specific esterases, which are members of the signal transduction

pathway (*Methods in Enzymology*, Vol. 197, E. Dennis, editor, Academic Press, NY) and are present in the circulation and on target tissues. These enzymes will then remove the phospholipid moiety and liberate the original compound with its biological activity intact. The above-mentioned enzymes are specific for phosphocholine; other esterases of the
5 signal transduction system would hydrolyze the other phosphoesters (*Methods in Enzymology*, Vol. 201, T. Hunter, Academic Press, NY, Beth Sefton, editor). In this manner, addition of phospholipid is expected to protect compounds from first-pass inactivation in the liver and allow them to reach their sites of action in the blood or in peripheral tissues.

10 Pharmaceutical agents suitable for use in the present invention include, without limitation, lipophilic compounds that exhibit poor solubility in biological fluids, as well as compounds that are rapidly metabolized in the liver to hippurate, glucuronate, or other derivatives. Non-limiting examples of suitable compounds include those that are not presently utilized in pharmaceutical applications, in particular as orally administrable
15 agents, because of problems with solubility, uptake, and/or metabolism. The only requirements for an agent to be used in the present invention are 1) the presence of a free alcohol functional group to which a phospholipid may be attached, and 2) the susceptibility of the resulting phosphodiester bond to cleavage by phospholipase C, sphingomyelinase or other mammalian esterases.

20 Examples of pharmaceutical agents suitable for use in the present invention include without limitation steroids, catecholamines such as epinephrine or norepinephrine, prostaglandins such as prostaglandin E1 or E2, leukotrienes such as leukotriene B4, C4 or D4 and peptides. Peptides for use in the present invention are those which contain serine or threonine and preferably should not be longer than 10-15 amino acid residues
25 in length such as Leutinizng Hormone Releasing Hormone (LHRH) (a 10 amino acid peptide) and its analogues. Preferred starting compounds or pharmacological agents include testosterone (available from Sigma, St. Louis, MO), etiocholanolone (Sigma), estradiol (Sigma), estrone (Sigma) and dehydroepiandrosterone (Sigma). These steroids have only limited activity when administered orally.

30 In an alternative embodiment of the present invention antibiotics, such as cephalosporin P1, can be conjugated to phospholipids in order to increase its aqueous solubility and decrease its metabolism on the first pass through the liver and excretion on the first pass through the kidney. Non-limiting examples of compounds for use in this

embodiment of the present invention include cephalosporin P1 (isolated as described in Burton et al., *Biochem. J.* 50:168-174, 1951; Halsall et al., *Chem. Comm.*, pp. 685-687, 1966), fusidic acid (commercially available from Sigma), and helvolic acid (commercially available from Sigma). Use of these antibiotics has been limited because
5 of an inability to development therapeutic serum and tissue levels in recipient mammals and, perhaps, because of the ease of development of resistance. The apparent resistance may be caused by induction of metabolic enzymes as occurs with other steroidal therapeutic agents.

Non-limiting examples of additional substances for use in the present invention
10 containing a free alcohol group include the steroidal substances mentioned above (DHEA, eticholanolone, testosterone, estradiol, estrone, catecholamines, etc.), the antibiotics mentioned above, aglycones including cardiac glycosides, such as digoxigenin (commercially available from Sigma), digitoxigenin (commercially available from Sigma), ouabagenin (commercially available from Sigma) and salicylic acid (commercially
15 available from Sigma).

Presented below is a further list of non-limiting examples of compounds for use in the present invention. Following the name of the compound, presented in parentheses is the number assigned to the compound in the Merck Index, 1996, 12th Edition. Menadiol (5873), Metronidazole (6242), Clindamycin (2414), Pentaerythritol
20 Tetranitrate (7249), Mesalamine (5964), β -Tocopherol (9632), γ -Tocopherol (9633), δ -Tocopherol (9634), Roxindole (8432), Vitamin E (10159), Styramate (9027), Strophanthidin (9015), Vitamin A (10150), Vitamin D₂ (10156), Vitamin D₃ (10157), Vitamin A₂ (10151), Calcitriol (1681), Diflunisal (3190), Clavulanic Acid (2402), Retinoic Acid (8333), Mazindole (5801).

25 A compound particularly well-suited for use in the present invention is the cyclic Urea-based HIV-1 protease inhibitor DMP-323 (*J. Med. Chem.* 39:2156-2169, 1996). Due to its low aqueous solubility investigators found that there was variability in the compounds bioavailability upon administration to patients and inconsistent efficacy. Addition of a phospholipid moiety is expected to improve its therapeutic use.

30 Other compounds well-suited for use in the present invention include aglycones from cardiac glycosides such as digoxigenin, digitoxigenin and ouabagenin (all commercially available from Sigma, St. Louis, MO.).

In addition to increasing the solubility of the above-identified compounds, the primary effect of conjugation to a phospholipid moiety to the following water soluble compounds is expected to be an increased half-life, that is to say, they will be long-acting forms of the parent compounds. Non-limiting examples of such compounds include

5 Isoproterenol (5236), Propranolol (8025), Methyldopa (6132), Epinephrine (3656), Codine (2525), Codine Phosphate (2528), Acetaminophen (45), Aspirin (886).

The conjugated therapeutic agents will be at least ten times more water soluble than the original alcohol. This will increase their bioavailability and decrease their metabolism to, e.g., the 3-glycoside in the case of steroids, which should be a major

10 excretion pathway. The decreased glycoside formation will be caused by the presence of the phosphoester at that site. The derivative is not expected to be active prior to hydrolysis of the phospholipid group. The present inventor has found that lymphocytes have an enzyme on their cell membrane that cleaves phosphocholine from other compounds (for example, sphingomyelin or lecithin) to release phosphocholine and the

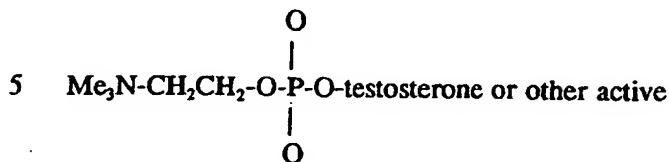
15 other ester conjugate (ceramide or diacylglycerol). The activity of this enzyme is stimulated ten-fold by TGF- α (data not shown). Without wishing to be bound by theory, it is believed that use of phospholipid-conjugated antibiotics of the present invention will lead to high concentrations of active agents at the site of an infection by the following mechanism. Lymphocytes are attracted to the site of an infection or inflammation where

20 they release TGF- α , which, in turn, stimulates phospholipid hydrolysis in other subtypes. This same process will lead to local release of an active form of the antibiotic from the phospholipid diester conjugate. Because of the response of the enzyme to local concentrations of TGF- α , there should be a correspondingly high local concentration of the antibiotic. This will lead to effective therapy and lower toxicity.

25 According to the present invention, starting compounds may be converted to phospholipid derivatives using any methods that are known in the art. In one preferred embodiment, phosphocholine (obtainable from Sigma Chemicals, St. Louis, MO) is reacted with a soluble carbodiimide, preferably 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, Sigma) in an active ester condensation

30 reaction. This carbodiimide is preferred because it, similar to phosphocholine, is water-soluble. The active phosphoester intermediate is then reacted with a pharmaceutically active agent to yield the desired phosphocholine ester. The reaction is shown in Example 1 below. Phosphocholine in water is reacted with EDAC to yield the active ester. This

is then reacted with, e.g., testosterone or other biologically active starting compounds etc., to yield the final product



or other active esterification product. The product is expected to be essentially water-soluble and thus easily separated from the starting compound by conventional extraction and/or separation methods e.g. Flash Chromatography, Thin Layer Chromatography, High Performance Liquid Chromatography (HPLC) and the like, as is known to those of ordinary skill in the art.

Alternate methods for synthesis of phosphocholine derivatives include phosphorylation of the steroid, peptide, etc. with DPPP to give a phosphate ester, e.g., testosterone phosphate, which is coupled to choline using EDAC as the complexing agent.

Alternately, the alcohol ("drug") may be reacted with phosphorous oxychloride and the aminoalcohol component added in excess. In this way all of the unreacted phosphorous oxychloride will be used up. The phosphochloride ester intermediate can also be isolated and reacted as a second step with the amino-alcohol component (choline, etc.). The final products can be purified by HPLC.

The phospholipid derivatized drugs of the present invention are expected to demonstrate enhanced biological activities, increased bioavailability and increased aqueous solubility. For example, etiocholanolone is metabolized by formation of the glucuronide in the liver of a mammal. After oral administration, about 99% of all free etiocholanolone is inactivated on each pass through the liver. When etiocholanolone is orally administered, it is absorbed in the gastrointestinal tract and transported via the portal circulation directly to the liver. Subsequently, only a fraction of a percent of the administered drug is biologically available for function. In contrast, phosphocholine-conjugated etiocholanolone may bind to form Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) cholesterol and is not expected to be degraded on first passage through the liver. In its phosphocholine-derivatized form, it is believed that about 80% of the etiocholanolone would not be metabolized at each pass. When the phosphocholine moiety is removed by an esterase, such as phospholipase C, sphingomyelinase, etc., then the parent compound will be available for binding and function in the target tissue.

Glucuronidation would only occur on its return to the liver after removal of the phosphocholine moiety.

The phospholipid-conjugated compounds of the present invention may be administered therapeutically by any route known in the art, e.g., orally, intravenously, 5 intramuscularly, subcutaneously, by inhalation or in aerosol form, and topically. The present invention is particularly applicable to compounds that, in their unconjugated state, cannot be effectively administered by the oral route.

The phospholipid-conjugated compounds of the present invention can be tested for efficacy as follows. A starting compound, and its phospholipid derivative, may be 10 administered by any of the above routes to a test animal, e.g., rat, mouse, rabbit, guinea pig, and the like. Serum samples are then collected at increasing times after administration, and the levels of the starting and conjugated compound are assayed and compared. It will be understood by those skilled in the art that the method of assay will depend upon the starting compound. In the case of steroids or peptides, High- 15 Performance Liquid Chromatography, Thin-Layer Chromatography, or immunoassay may be used to quantify serum levels. When the starting compounds are gonadal steroids, it may also be necessary to gonadectomize the test animals prior to drug administration, so as to suppress endogenous production of the test compound. Successful compounds are those whose serum level is increased significantly by administration of the phospholipid 20 derivative relative to administration of the starting compound or by their ability to reach therapeutically significant serum levels when administered by an alternate route, e.g. orally.

In a second phase, the starting compound and its phospholipid derivative will be administered to test animals, and the physiological effect of the compounds assayed 25 over time. For example, for etiocholanolone and its phospholipid derivative(s), rate of weight gain and changes in basal metabolic rate are measured. Estradiol, estrone and their phosphocholine derivatives will be administered by gavage to ovariectomized mice or rats and changes in uterine weight, breast development and estradiol blood levels will be measured. Testosterone and its phosphocholine derivative will be administered orally 30 to castrate mice or rats and changes in seminal vesicles, prostate size, and levator and muscle will be determined. Theophylline and its phosphocholine derivatives will be given orally to rats and the blood levels over the next 6 hours will be determined. From these tests, the degree to which the phospholipid derivatives are more potent than the

underivatized parent compound will be determined, i.e., the same response will be achieved with a smaller dose of the derivatized compound than the parent compound. This will be a measure of greater potency. Successful compounds are those whose functional endpoints are significantly lower for phospholipid derivatives than for the starting compounds.

In a preferred embodiment of the present invention, testosterone is converted to testosterone-17-phosphocholine, estrone is converted to estrone-3-phosphocholine and estradiol is converted to estradiol-3-phosphocholine or estradiol-17-phosphocholine. In like manner, theophylline is converted to theophylline phosphocholine. These compounds will frequently be given as replacement therapy for various hormone deficiencies and as pharmacological therapies in other cases. Theophylline is given to treat asthma, estradiol is administered to treat osteoporosis, etiocholanolone is given as a haemopoietic agent, to promote weight loss and to reduce diabetic blood sugar levels. Similar derivatives could also be used to provide enhanced levels of epinephrine.

The present invention also provides pharmaceutical formulations and dosage forms comprising the phospholipid-derivatized drugs of the present invention. The pharmaceutical formulations of the present invention may also include, as optional ingredients, pharmaceutically acceptable vehicles, carriers, diluents, solubilizing or emulsifying agents, and salts of the type well known to those of ordinary skill in the art.

The phospholipid-derivatized drugs of the present invention can be incorporated into pharmaceutical formulations to be used to treat mammals. Pharmaceutical formulations comprising the phospholipid-conjugated drugs of the present invention as at least one of the active ingredients, would in addition optionally comprise pharmaceutically-acceptable carriers, diluents, fillers, salts and other materials well-known in the art depending upon the dosage form utilized. For example, preferred parenteral dosage forms may comprise a sterile isotonic saline solution, 0.5 N sodium chloride, 5% dextrose and the like. Methyl cellulose or carboxymethyl cellulose may be employed in oral dosage forms as suspending agents in buffered saline or in cyclodextran solutions to enhance solubility.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose or dosage form need not in itself constitute an effective amount for the various usages of the phospholipid-derivatized drugs of the present

invention since the necessary effective amount can be reached by administration of a plurality of such dosage forms.

The following examples are intended to further illustrate the present invention without limiting it thereof.

5

EXAMPLE 1 : SYNTHESIS OF PHOSPHOCHOLINE DERIVATIVES

Method 1

Phosphocholine (Sigma) (0.1 mol) is stirred in pyridine
10 (Fisher, VWR) (100 ml) with 0.1 mol of morpholine (Sigma) and 0.1 mol of DDC (Sigma) for 6 hours under nitrogen or argon. At this point the reaction complex is stirred while 0.1 mol of steroid (etiocholanolone, estradiol, testosterone) are added. After stirring for an additional 3 hours the reaction mixture is diluted with 1 liter of ice water. The insoluble N,N' dicyclohexylurea is removed by filtration and the aqueous fraction is
15 extracted with 4 X 0.5 volumes of ethyl acetate. The ethyl acetate is washed with saturated brine (0.1 vol) to remove the pyridine and dried over sodium sulfate. The solvent is removed by filtration and the product isolated by LH-20 column chromatography or by preparative HPLC.

20

Method 2

Phosphocholine (0.1 mol), steroid (0.1 mol) as above and DCC (0.12 mol) are stirred in 100 ml of pyridine (VWR) at 80° for 6 hours under nitrogen. The solution is diluted with 600 ml of water and processed as described above.

25

Method 3

Testosterone or other steroid, prostaglandin, etc. (0.1 mol) is reacted with POC₁, in pyridine to yield the steroid phosphate. This product after drying in pyridine will then be reacted with 0.1 mol of EDAC at a rate just sufficient to maintain the pH at 7.0. The product is then purified as described above.

30

The compounds will then be analyzed by HPLC to determine purity of the reaction product, by NMR to verify the structure and by UV and IR spectra to determine their identity. Treatment with a phosphodiesterase will then be used to cleave the diester to further establish the structural identity.

**EXAMPLE 2: PHARMACOKINETICS OF TESTOSTERONE AND ITS
PHOSPHOCHOLINE DERIVATIVE**

5 The phosphocholine derivatives of testosterone (about 5 mg) is dissolved in 20 ml of buffered saline or in 20 ml of 40% cyclodextran in saline and given orally to human volunteers. Alternatively, testosterone (5 mg) is suspended in a carboxymethyl cellulose suspending media, vortexed and then given orally. Blood samples will be taken at 30, 60, 120, 240, 360 and 720 minutes post-administration and collected in green top tubes. The
10 blood samples are centrifuged and the plasma collected and stored as aliquots in microfuge tubes. The samples are then analyzed for testosterone in duplicate using a standard RIA kit (Diagnostics Products Corp., Tarzana, CA).

15 **EXAMPLE 3: MEASUREMENT OF BIOACTIVITY OF PHOSPHOCHOLINE
DERIVATIVES**

 The bioactivity of orally administered estradiol and estradiol phosphocholine will be determined in ovariectomized mice or rats. In addition, other animals will be briefly anesthetized and the steroid phosphocholine derivative or the free steroid will be
20 administered intraperitoneally (IP). After 2 days the animals are sacrificed and the 4th and 9th inguinal breast tissue will be isolated. At the same time the uteri will be isolated and weighed. It is expected that the phosphocholine derivatized steroid will be more active than the parent compound when administered orally and by IP injection.

 Estradiol and its phosphocholine derivative will also be administered by gavage
25 to ovariectomized mice or rats and changes in uterine weight, breast development and estradiol blood levels will be measured. Estradiol will be measured with an RIA kit from Diagnostics Products Corp. (Tarzana, CA).

 Testosterone and its phosphocholine derivative will be administered orally to castrate male mice or rats and changes in seminal vesicles, prostate size, and levator ani
30 muscle will be determined. Testosterone blood levels will also be measured by RIA using a kit from Diagnostics Products Corp. (Tarzana, CA). The compounds will also be characterized by UKV. Responses will also be measured after IP injection.

 Theophylline and its phosphocholine derivatives will be given orally to rats and the blood levels of theophylline will be measured over the next 6 hours using an RIA kit
35 (Diagnostics Products Corp., Tarzana, CA).

From these tests, the degree to which the phosphocholine derivatives are more potent than the underivatized parent hormone can be determined; i.e., the same response will be achieved with a smaller dose of the derivatized compound than the 25 parent compound. This will be a measure of greater potency.

5

**EXAMPLE 4: SYNTHESIS OF DEHYDROEPIANDROSTERONE (DHEA)-
PHOSPHOCHOLINE DERIVATIVE**

A dehydroepiandrosterone(DHEA)-phosphocholine derivative was synthesized
10 as follows: 1 mg of phosphocholine (calcium salt; Sigma Chemical, St. Louis, MO) was
dissolved in 0.5 ml of formamide (Cat # S-7503; Lot # 55HO257; Sigma Chemical) and
0.5 ml of pyridine (Cat # P-4036; Lot # 55HI489; Sigma Chemical). 0.025 mCi of
(1,2,6,7 ³H(n)-Dehydroepiandrosterone (Cat # NET814; Lot # 3146097; 89.2 Ci/mmol;
Dupont, NEN Products, Boston, MA) in 0.025 ml of ethanol was added. The reaction
15 was catalyzed by the addition (as the dry solid) of 5 mg of dicyclohexylcarbodiimide (Cat
D-3129; Lot # 34hO647; Sigma Chemical). The reaction mixture was incubated
overnight at room temperature. In the morning, 9 ml of water was added and the mixture
extracted 3 times with 10 ml portions of benzene. The benzene extracts were combined
and aliquots of both phases were counted in a scintillation counter. The results are set
20 forth below:

Aqueous Phase	10,729 cpm (0.01ml)
Benzene Phase	1,121 cpm (0.01ml)

The aqueous layer was re-extracted with benzene. The second benzene
25 extraction yielded 272 cpm (0.01 Ml) as a confirmation.

Free DHEA starting material would have been extracted quantitatively into
benzene with this protocol. The observation that the reaction product remains in the
aqueous phase confirms its increased hydrophilic characteristics.

30 **Example 5: DHEA-3-PHOSPHOCHOLINE: SYNTHESIS AND BIOACTIVITY**

DHEA-phosphocholine (DHEA-PC) was synthesized by sequential reaction of
DHEA, choline, and water with phosphorous oxychloride. The synthetic product had the
same HPLC retention time and the same mass-spectrum as did the endogenous, actual
compound. It was hydrolyzed by neutral sphingomylenase, but not by acidic

sphingomylenase. When human serum extracts were analyzed, mass fragments were detected at the same retention time as synthetic material. When DHEA-PC was administered to mice, it potentiated dinitrochlorobenzene-induced sensitization as detailed below.

5

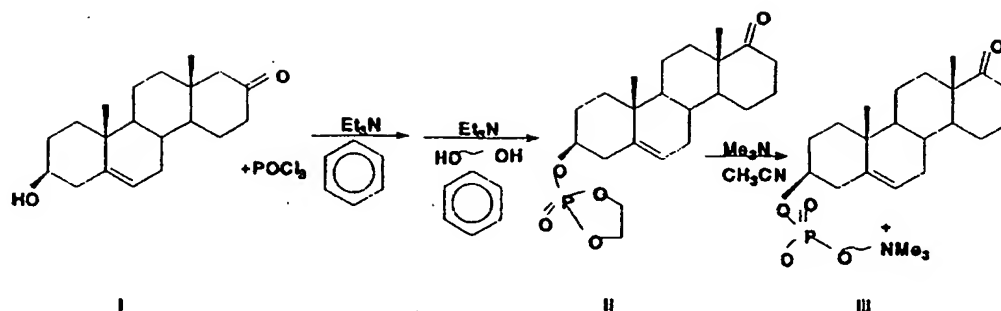
Example 6: DHEA-PC POTENTIATES DNCB-INDUCED IMMUNOLOGICAL SENSITIZATION

10 The effects of DHEA-PC on cutaneous contact hypersensitivity was studied. In this study, mice (Balb/c) were immunologically challenged with DNCB (2% in ethanol) applied to a 2 cm area on the back. The steroid was injected subcutaneously (100 µg/day/mouse) throughout the twelve-day study period. Ears were rechallenged with DNCB (1% in ethanol) on days 7-12 and swelling was measured daily in order to evaluate
15 the effect on the immune system.

 During days 7-12, DHEA-PC enhanced the cutaneous hypersensitivity immune response similar to native steroids (DHEA and DHEA-sulfate). The response to these three hormones was not suppressed by dexamethasone even though, when administered by itself, dexamethasone suppressed the immune response below the control. This shows
20 that all three hormones induced a similar, high level response.

Example 7: NOVEL PHOSPHOCHOLINE SYNTHETIC METHOD

 DHEA (I) (82.0g, 0.284 mol, Steraloids, Inc., Wilton, NH) was dissolved in
25 a 5 L, 3 necked, round bottom flask in dry benzene (1.5 L, Fisher, Pittsburgh, PA). Gentle heating was applied to facilitate the process. Triethylamine (30.3g, 41.6 mL, 0.30 mol, Aldrich, Milwaukee, WI) was added all at once. After the reaction was cooled down to room
temperature, oxyphosphorus trichloride (43.6 g, 26 mL, 0.284 mol, Fluka, Ronkonkoma,
30 NY) was added in one portion. The mixture was stirred under nitrogen overnight (12 hours). The precipitate was filtered off via canula transfer under nitrogen, and washed once with dry benzene (300 mL). To the combined clear benzene solution was added ethylene glycol (18.6 g, 0.30 mol, Aldrich) and triethylamine (61 g, 0.60 mol, Aldrich). The mixture was stirred rapidly for 16 hours at room temperature. Thin layer



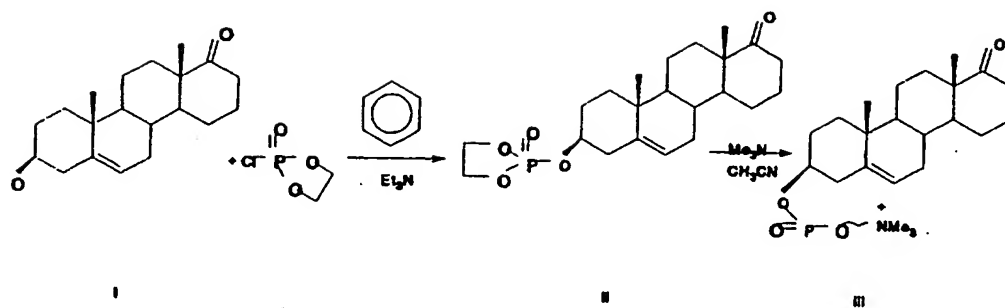
chromatography (TLC) (Silica gel,

5

developed with ethyl acetate, Fisher) showed almost complete conversion. The newly formed precipitate was separated on a Buchner funnel and washed three times with hot dry benzene (800 mL total). The combined filtrates were evaporated to dryness on a rotary evaporator (Buchi, Fisher). The intermediate (II) was a white solid and used for the next
 10 step without further purification. An additional amount of the intermediate (II) was obtained from the solid retained by the Buchner funnel by resuspension in water and vacuum filtration. The combined collected solid was air dried. The overall yield of the crude intermediate (II) was virtually quantitative (110 g).

The crude II (2.9 g) was suspended in acetonitrile (25 mL, Fisher Chemicals).

15 While the mixture was stirred at 50–60°C with the pressure maintained through a balloon, trimethylamine (Aldrich) was introduced as the gas. After the conversion was complete, as indicated by TLC analysis, the mixture was vacuum filtered, washed repeatedly with acetonitrile and then air dried. The yield was 75% (2.5 g). LC-mass spectroscopy (Micromass, Beverly, MA) showed a major peak at $R_f=9.8$ min with mass of 454 daltons
 20 (M+H), as predicted. DHEA (8.75g, 0.031 mol) was dissolved in benzene and triethylamine (4.45 mL) was added. 2-chloro-1,3,2-dioxaphospholane-2-oxide (4.54g, 0.032 mol, Aldrich) was then added at room temperature. The reaction mixture was stirred until complete conversion of DHEA to II occurred. The reaction was monitored by TLC (silica gel, ethyl acetate). After filtration, the solid was washed with dry
 25 benzene. The combined benzene solution was concentrated to give a white solid (II) and



used without further purification.

A sample of II (0.75 g) prepared as above was suspended in acetonitrile (10 mL) and stirred with heating. Trimethylamine was introduced as a gas while the pressure was regulated with a balloon attached to one of the necks of the flask. When TLC (silica gel, ethyl acetate) showed the disappearance of II, the addition of gas was stopped. The product (III) was collected by vacuum filtration, washed with additional acetonitrile and air dried. The yield was 0.72g (83%).

WHAT IS CLAIMED IS:

- 1 1. A method for increasing the aqueous solubility of
2 a pharmaceutically active agent, comprising the steps of
3 conjugating said agent to a phospholipid
4 moiety, wherein said phospholipid moiety is selected from the group consisting of
5 phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-
6 phosphoethanolamine and N, N-dialkyl-phosphoethanolamine and
7 recovering said pharmaceutically active agent conjugated to said
8 phospholipid.
- 1 2. The method of claim 1, wherein said agent is selected from the group
2 consisting of a steroid, peptide, prostaglandin, catecholamine, and a leukotriene.
- 1 3. The method of claim 1 wherein said agent is an antibiotic selected from
2 the group consisting of cephalosporin P1, fusidic acid and helvolic acid.
- 1 4. The phospholipid conjugated pharmaceutically active agent produced
2 by the method of claim 1.
- 1 5. A pharmaceutical formulation comprising a phospholipid-conjugated
2 active agent wherein said agent is selected from the group consisting of testosterone,
3 estrone, estradiol, etiocholanolone, and dehydroepiandrosterone and a pharmaceutically-
4 acceptable carrier or diluent wherein said phospholipid is selected from the group
5 consisting of phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-
6 phosphoethanolamine and N, N-dialkyl-phosphoethanolamine.
- 1 6. A pharmaceutical formulation for treating a mammal suffering from
2 asthma comprising an isolated phospholipid derivative of theophylline and a
3 pharmaceutically acceptable carrier or diluent wherein said phospholipid is selected from
4 the group consisting of phosphoserine, phosphotyrosine, phosphoethanolamine, n-
5 monoalkylphosphoethanolamine and N, N-dialkyl-phosphoethanolamine.

1 7. A pharmaceutical formulation comprising an isolated phospholipid
2 derivative of an antibiotic selected from the group consisting of cephalosporin PI, fusidic
3 acid and helvolic acid, and a pharmaceutically acceptable carrier or diluent.

1 8. A pharmaceutical formulation comprising a phospholipid-conjugated
2 pharmaceutically active agent wherein said agent is selected from the group consisting of
3 digoxigenin, digitoxigenin, ouabagenin and salicylic acid, and a pharmaceutically
4 acceptable carrier or diluent.

1 9. A pharmaceutical formulation comprising a biologically-active
2 phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from
3 the group consisting of Menadiol, Metronidazole, Clindamycin, Pentaerythritol
4 Tetranitrate, Mesalamine, β -Tocopherol, γ -Tocopherol, δ -Tocopherol, Roxindole, Vitamin
5 E, Styramate, Strophanthidin, Vitamin A, Vitamin D₂, Vitamin D₃, Vitamin A₂,
6 Calcitriol, Diflunisal, Clavulanic Acid, Retinoic Acid, and Mazindole and a
7 pharmaceutically acceptable carrier or diluent.

1 10. A pharmaceutical formulation comprising a phospholipid-conjugated
2 derivative of DMP-323 and a pharmaceutically acceptable carrier or diluent.

1 11. A pharmaceutical formulation comprising a phospholipid-conjugated
2 pharmaceutically active agent wherein said agent is selected from the group consisting of
3 Isoproterenol, Propranolol, Methyldopa, Epinephrine, Codeine, Codeine Phosphate,
4 Acetaminophen, and Aspirin, and a pharmaceutically acceptable carrier or diluent.

1 12. A composition of matter comprising an isolated phospholipid derivative
2 of an antibiotic selected from the group consisting of cephalosporin PI, fusidic acid and
3 helvolic acid.

1 13. A composition of matter comprising a phospholipid-conjugated
2 pharmaceutically active agent wherein said agent is selected from the group consisting of
3 digoxigenin, digitoxigenin, ouabagenin and salicylic acid.

1 14. A composition of matter comprising a biologically-active phospholipid-
2 conjugated pharmaceutically active agent wherein said agent is selected from the group
3 consisting of Menadiol, Metronidazole, Clindamycin, Pentaerythritol Tetranitrate,
4 Mesalamine, β -Tocopherol, γ -Tocopherol, δ -Tocopherol, Roxindole, Vitamin E,
5 Styramate, Strophanthidin, Vitamin A, Vitamin D₂, Vitamin D₃, Vitamin A₂, Calcitriol,
6 Diflunisal, Clavulanic Acid, Retinoic Acid, and Mazindole.

1 15. A composition of matter comprising a phospholipid-conjugated
2 derivative of DMP-323.

1 16. A composition of matter comprising a phospholipid-conjugated
2 pharmaceutically active agent wherein said agent is selected from the group consisting of
3 Isoproterenol, Propranolol, Methyldopa, Epinephrine, Codine, Codine Phosphate,
4 Acetaminophen, and Aspirin.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17640

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 31/70, 31/675, 31/335.

US CL :514/12, 18, 43, 50, 81, 449.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 18, 43, 50, 81, 449.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,194,654 A (HOSTETLER et al.) 16 March 1993, column 2, lines 43-57.	1-16
Y	US 5,529,989 A (PETTIT et al.) 25 June 1996, column 3, line 58 to column 4 line 40.	1-16
Y	US 5,411,947 A (HOSTETLER et al.) 02 May 1995, entire document.	1-16
Y	US 5,484,809 A (HOSTETLER et al.) 16 January 1996, entire document.	1-16

<input type="checkbox"/> Further documents are listed in the continuation of Box C.	<input type="checkbox"/> See patent family annex.
<p>* Special categories of cited documents:</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*B* earlier document published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p>	<p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>*A* document member of the same patent family</p>
Date of the actual completion of the international search 11 DECEMBER 1997	Date of mailing of the international search report 13 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Theodore J. Criares</i> THEODORE J. CRIARES Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17640

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN:

search terms: prodrug, phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-phosphoethanolamine, N, N-dialkyl-phosphoethanolamine.